

LABORATORY NOTEBOOK

Notebook No.: _____

Assigned to: ARTHUR BRANSTROM

Date: _____

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Exhibit 9

Construction of new plasmid pAB103

pMB1 ORI of replication

pSC101 ORI of replication

ASO (Bgl II Fragment) FROM pTA3167

Plasmid pAB102 WAS DIGESTED \pm Bgl II to remove E. coli ASO & replaced \pm pTA3167 ASO

Ligated DNA was transformed into X6097 cells to isolate plasmids of interest.

Km^r cassette was inserted @ the PAMH2 restriction site to check for in vitro plasmid stability.

1321 [OVERNIGHT GROWTH IN LB ~~OR~~ LB ARO]

@ 12 HR timepoint, 96/100 Km^r

@ 50 GENERATIONS (6 HRS IN FRESH Km^r MEDIA) 95/100 Km^r

2000 [OVERNIGHT CULTURES GROWN IN LB ARO]

@ 12 HRS 67/100 Km^r

@ 50 GENERATIONS 55/100 Km^r

1321 LYSO containing plasmids pAB102 or pAB103 were given

to D. Sizemore to pass them mice (BALB/C - orally)

- HIV genes VIF & GAG are cloned into the PAMH2 & SRI sites of plasmid pAB103 (UNDER control of pluc⁺)

Expression was detected for both by using rabbit sera or human sera respectively.

- The VIF & GAG constructs were given to D. Sizemore to pass them the mice again.

While recovery varied from that observed with pAB102 constructs, (i.e. lower recovery of bacteria from the spleens, appears to be slower in vivo growth rate, isolates were collected that expressed levels of VIF & GAG that were as high as what went in. Only VIF was overexpressed in pAB102 isolated from spleens.

Plasmids from low expressing & high expressing strains are isolated, & will be used to complement back plasmid cured strains (HIGH & LOW level expressing) to determine if the mutation in vivo is occurring @ the plasmid or chromosomal level

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Author: A. Brantner

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PROJECT ENV (MN) III_B

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- 2 different regions of the ENV gene are PCR amplified.
- the regions chosen are conserved for the primer sequences between MN & III_B.
 - Amplification from both HIV genomes amplified similar size fragments. Sequences were pulled from the GCG database.
 - These 4 PCR products are BAMHI/SAL1 digested & cloned into pUC18 (β-gal indicator + overexpression to determine if the envelope constructs are being translated).

Arthur A. Brantner

NOTE: TEXT PRINTED FOR FUTURE "READABILITY"

- 2 ENV REGIONS WERE ISOLATED, CLONED INTO pUC18:
- ENTIRE gp140 (FROM BOTH MN & III_B)
 - V₃ LOOP REGION (FROM BOTH MN & III_B)
 - III_B ENVELOPE A.I. FROM # 263-610
 - V₃ LOOP CONTAINING CLONED DESIGNATED ENV_{MN}A1 OR ENV_{III_B}
- THE ENTIRE GP140 APPEARS TO BE FULLY EXPRESSED COMPARED TO THE A1 REGION.

- EXPRESSION WAS DETECTED BY HUMAN SERA

Arthur A. Brantner

ANALYSIS OF 1321 DASD, PAB22 CONSTRUCTS THAT WERE AFTER COLLECTION OF BACTERIA FROM MOUSE SPLEENS, HIV OR GAG EXPRESSION WAS ANALYZED WITH HIV ANTI-SERA (RABBIT), OR HUMAN SERA TO GAG. EXPRESSION WAS VARIABLE FOR BOTH ISOLATES, SOME BEING LOW LEVEL EXPRESSORS, AND SOME BEING COMPARABLE TO WILD-TYPE. IT APPEARS THAT THE DECREASED LEVELS OF EXPRESSION ARE DUE TO REDUCED PLASMID COPY NUMBER. ALSO PRIMERS WERE RETROD TO AMPLIFY ASD & ITS ENDOGENOUS PRIMERS, & CLONED UNDER THE CONTROL OF p_{lac} p_{lac} DID NOT RESULT IN A DECREASE IN ASD PRODUCTION.

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pAEC WAS CLONED INTO pA3103 RESULTING IN PLASMID
DESIGNATED pA3104. pAEC WAS CLONED VIA *EcoRI* &
SAL1 RESTRICTION SITES, & THE REINTRODUCTION OF ASD
(FLANKED BY *EcoRI* SITES). THIS CLONE CONTAINS:
pSC101 ORIGIN OF REPLICATION, pUC18 ORIGIN OF REPLICATION,
ROY CURTIS'S ASD FROM *S. Typhimurium*, pAEC PROMOTER &
BAMHI - SAL1 POLYLINKER.

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COMPLETE EAV (EPIG) REGIONS FROM BOTH HIV₁ & HIV₂ ARE CLONED INTO THE VARIOUS DELIVERY VECTORS. pAB102 & pAB103 ARE USED TO EXPRESS THE SEQUENCES, ALTHOUGH DETECTION WAS NEGLIGIBLE DUE EITHER TO THE LOWER COPY #'S OF THESE PLASMIDS COMPARED TO pUC18, OR TO THE INSTABILITY OF THE ~~gag~~ SEQUENCE.

Arthur A. Brantner

IN COLLABORATION = D. SZEMER, THE VIF WAS CLONED INTO PLASMID pAB104 & ASSAYED FOR EXPRESSION IN E. coli K6097. EXPRESSION IS \approx EXPRESSION FROM pAB102 OR pAB103 (pLac PROMOTER).

Arthur A. Brantner

THE ANTIBODY RECOGNITION FRAGMENT "TNRNPQ" WAS CLONED (AFTER SYNTHESIZING) INTO VARIOUS VECTOR CONSTRUCTS.

COMPLEMENTARY SEQUENCES:

		T	N	R	N	P	Q
FORWARD OLIGO		GATCGTACG	AAC	CGG	AAT	CCT	CAG
REVERSE OLIGO		CATGCTTG	GCC	TTA	GGA	GTG	CTAG
		MUTATED BAMHI SITE			CONSERVED BAMHI SEQUENCE		

THE DIFFICULTY IN DETECTING POSITIVE CLONES WAS CIRCUMVENTED BY CONSTRUCTING TNRNPQ SEQUENCES TO BE PROXIMATELY CLONED USING E. coli BAMHI RESTRICTION SITES. THE OLIGO WAS DESIGNED TO BE PLACED ON THE AMINO-TERMINAL END OF THE EXPRESSED PROTEIN.

FORWARD OLIGO	AATTGTG	GAT	ACG	AAC	CGG	AAT	CCT	CAG	GGG
REVERSE OLIGO	CA	CCA	TGC	TTG	GCC	TTA	GGA	GTG	CCCTAG

THESE OLIGOS AMPLIFIED & LIGATED INTO pAB18::VIF & THE BAMHI/E. coli SITES. EXPRESSION VIA 2A10 ANTIBODY WAS PRACTICALLY NON-EXISTENT ALTHOUGH VIF WAS BEING TRANSLATED DUE TO ITS REACTIVITY & VIF ANTISERUM.

Arthur A. Brantner

THE IMPORTANCE OF HAVING TNRNPQ ON THE NH₂ OR COOH TERMINAL END WAS EVALUATED USING VIF-TNRNPQ ENGINEERED SEQUENCES. THESE (VIF-TNRNPQ) CONSTRUCTS WERE VERY POORLY RECOGNIZED BY THE 2A10 ANTIBODY. THE PROBLEM APPEARS TO BE THE RESULT OF NOT ENOUGH COPIES OF THE TNRNPQ SEQUENCE FOR THE 2A10 ANTIBODY TO RECOGNIZE.

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A TANDUM REPEAT OF TURNPQ IS SYNTHESIZED AS A VIF FUSION OLIGO (127 bp) TO BE USED IN PCR AMPLIFICATION & VIF F FORTH. THIS RESULTED IN A VIF-[TURNPQ]₄ FUSION, WHICH WAS PCR DERIVED, & CLONED INTO BAM/SAL SITES OF pAB102 & pAB104. EXPRESSION WAS DETECTABLE, BUT NOT AS REACTIVE WHEN COMPARED TO VIF REACTED WITH RABBIT anti-VIF SERUM.

Arthur A. Brantner

THE [TURNPQ]₄ SEQUENCE IS USED TO TAG ENV EXPRESSED IN VARIOUS PLASMID CONSTRUCTS TO DETERMINE IF DIFFICULTY IN DETECTION IS DUE TO POOR EXPRESSION, OR THE LACK OF HIGH ACTIVITY/TITRAL ANTIBODY.

ENV_{MAN} & ENV_{INT} ARE CLONED FROM BOTH HIV-1 & HIV-2 UTILIZING CONSERVED SEQUENCES (ENTIRE GP120 TAGGED & RECON END OF PROTEIN). PCR AMPLIFIED DNA IS CLONED INTO BAMHI/SAL SITE OF pAB101, pAB102, & pAB103.

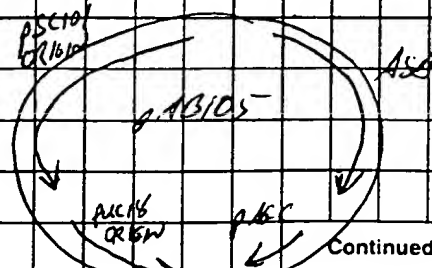
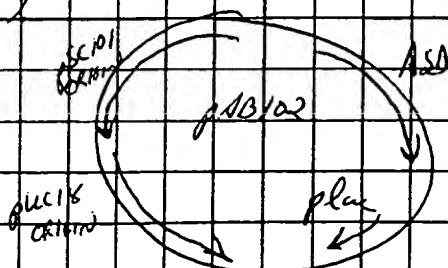
THE CONSTRUCTS WERE MORE UNSTABLE THAN MAN CONSTRUCTS, RESULTING IN LARGE DELETIONS OF THE PLASMIDS. NEITHER SET OF CONSTRUCTS EXPRESSED PROTEIN DETECTABLE BY 2A10 ANTIBODY.

Arthur A. Brantner

ASD FROM pAB102 WAS INTRODUCED INTO A PLASMID (pAB104) CONTAINING THE pUC ORIGIN OF REPLICATION. THIS E. COLI ASD (pAB105) HAD THE POTENTIAL OF BEING IN 2 POSSIBLE ORIENTATIONS. DOUBLE DIGESTS WITH BAMHI & HINDIII DETERMINED THAT ASD IN pAB105 IS IN THE SAME ORIENTATION AS 11-12.

Arthur A. Brantner

THE DIRECTION OF THE pUC ORIGIN OF REPLICATION WAS DETERMINED BY PCR ANALYSIS. THE DIRECTION OF REPLICATION CONVERGES TOWARDS THE PROMOTER (pLac in pAB102 OR pLac in pAB105).



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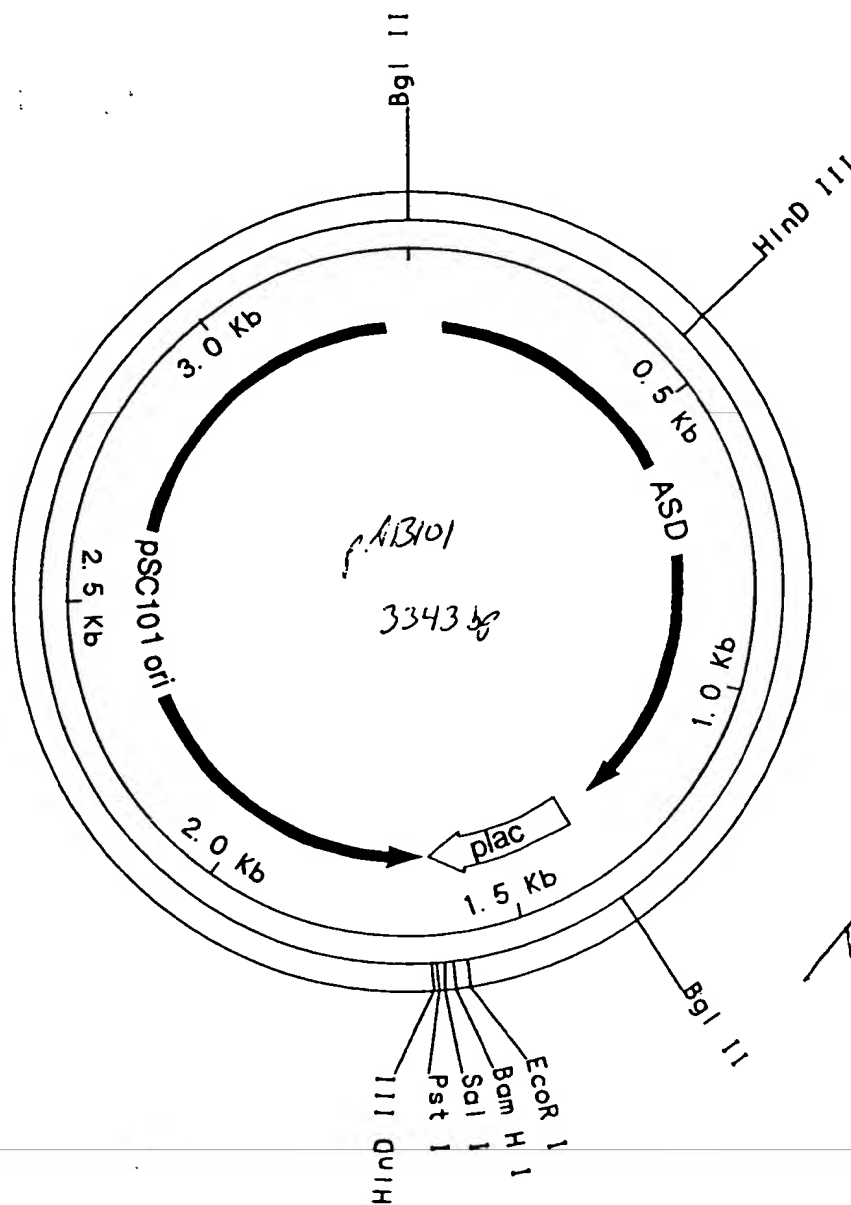
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Plasmid maps are constructed from the GCG data bank, & are made for all 5 plasmid constructs, pAB101 → pAB105, with subtle differences between various segments.



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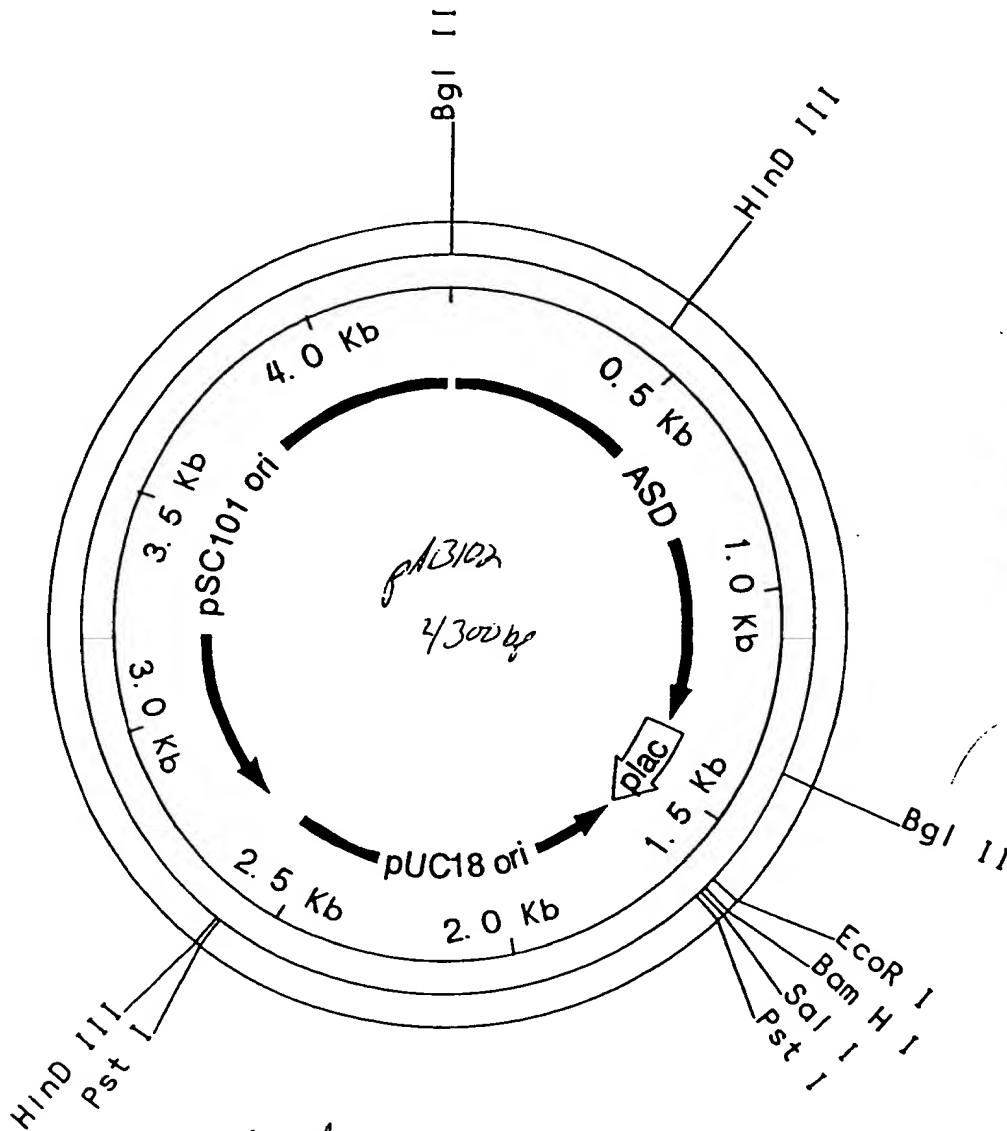
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Arthur A. Branton

11/13

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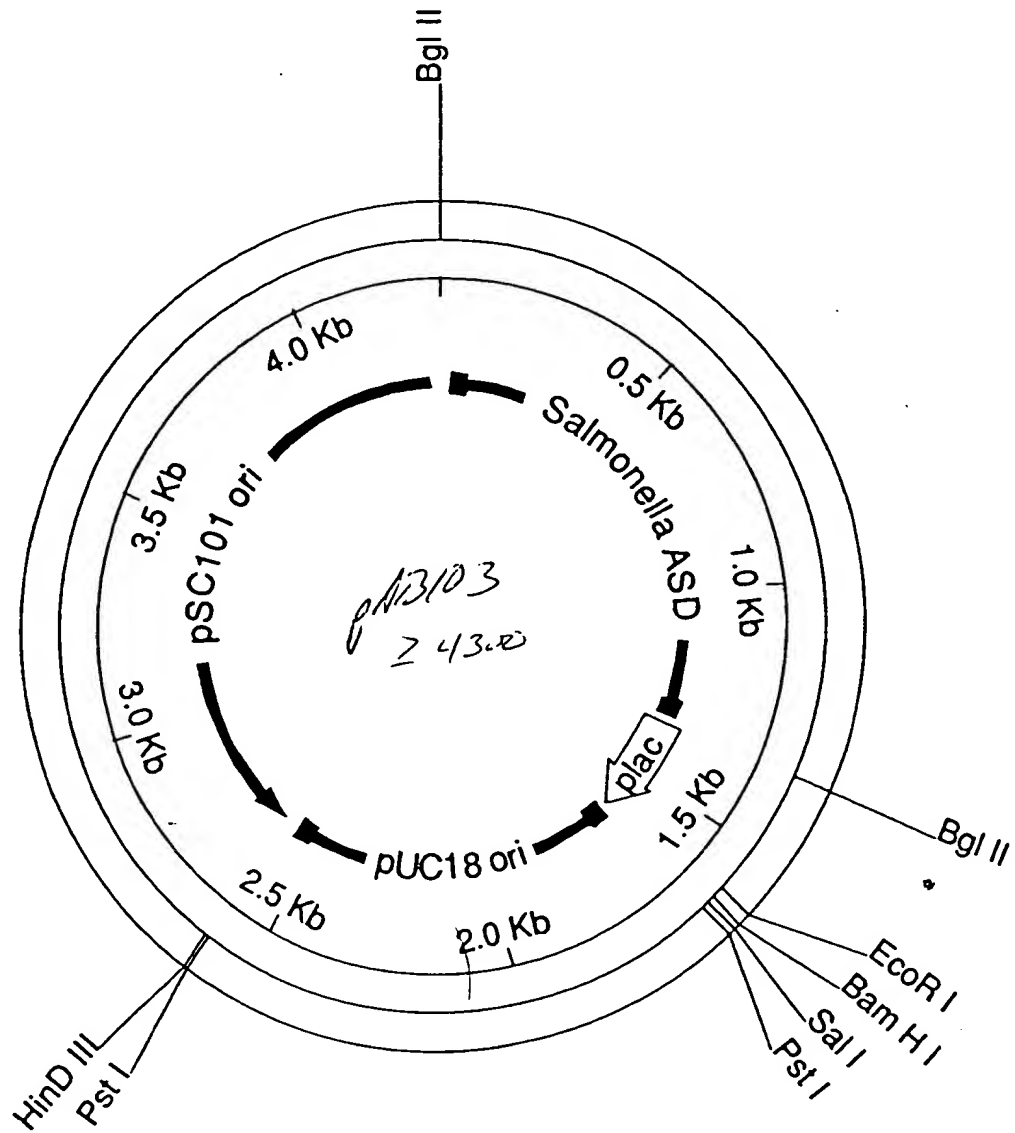
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Arthur A. Drenth

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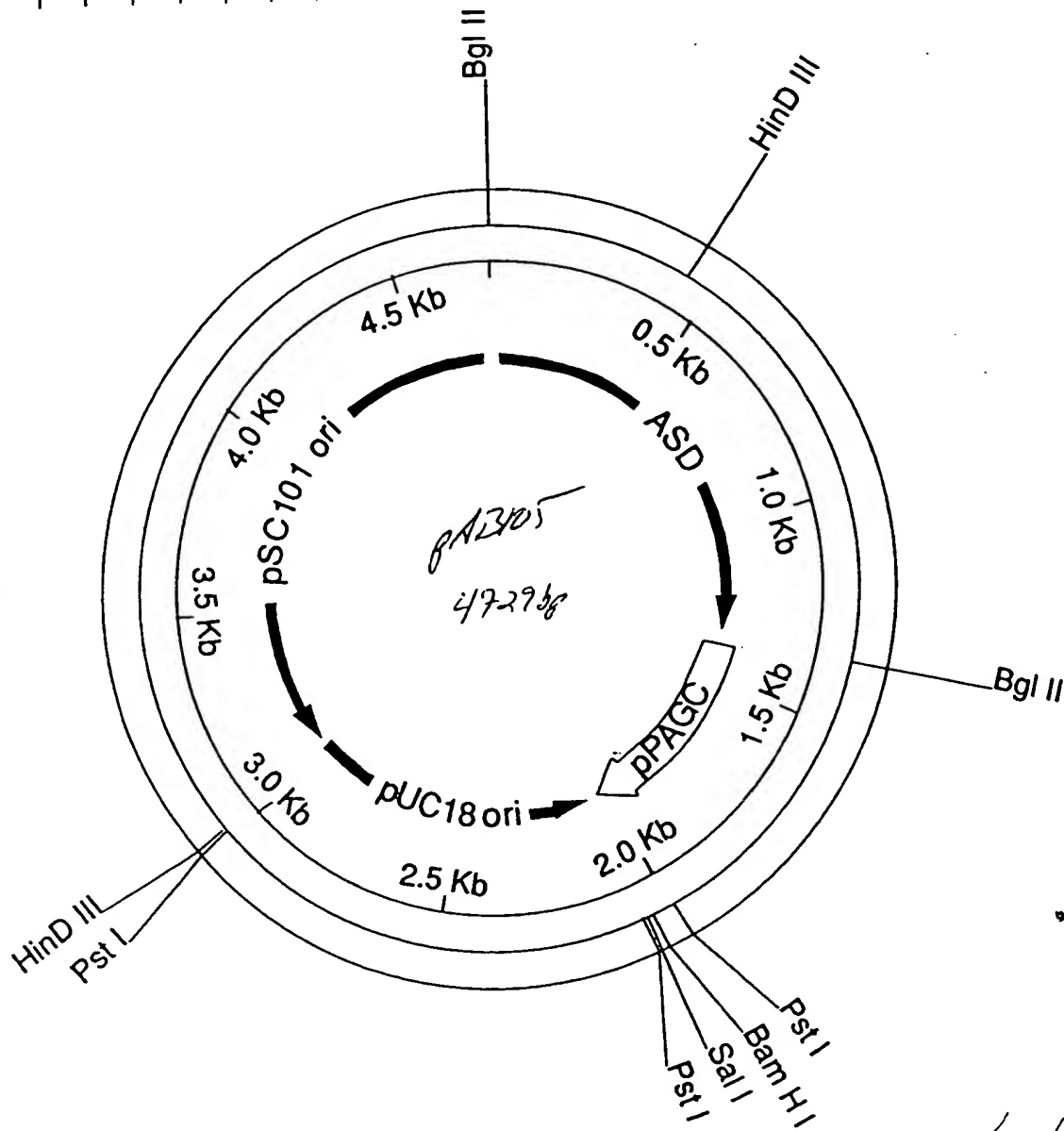
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Arthur A. Branstetter

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PRISON - A GENE FUSION WAS CONSTRUCTED FOR VIF USING PLASMID pRIT-2T (PHARMALIA). VIF PRIMERS WERE MADE TO ALLOW IN FRAME CLONING WITH BAMHI SITE, & UTILIZATION OF SALT SITE DOWNSTREAM OF VIF FOR DIRECTIONAL CLONING.

Arthur A. Brantner

(Fetal Genebank)
CHROMOSOMAL PROBS WERE DONE FOR 1321 AND CONSTRUCTED FOR ISOLATES CONTAINING PLASMID pAB101 → pAB105. THE PLASMID COPY #3 WERE COMPARED TO S. Typhimurium 1321 RELEVANT FROM MOTHER SPOONS THAT CONTAINED pAB103:GAG THAT WAS EXPRESSED POORLY, & pAB103:VIF THAT WAS ALSO WEAKLY EXPRESSED AS COMPARED TO WILD-TYPE BEFORE PASSAGE THREE MAKE.

Arthur A. Brantner

THE pRIT-2T SEQUENCE CONTAINS A PUTATIVE PROTEOLYTIC CLEAVAGE SITE AT POSITION #7774 bp. IF THE SITE WAS RECOGNIZED IN E. COLI OR SALMONELLA (OR SHIGELLA), THE EXPECTED PRODUCTS WOULD BE 27,805 KDa (7040-7774 aa) AND 11,182 KDa (7775-8083 aa). THIS DOES NOT APPEAR TO BE THE CASE.

Arthur A. Brantner

ELECTROPORATION FOR S. FLEXNERI 245FT WAS ASSAYED, & FOUND TO BE OPTIMAL FOR THE FOLLOWING CONDITIONS:

2.0 KVOLTS, 25 nF, 800 Ω. CELLS WERE PROTHIRED VIA THE PROCEDURE FOR "PROTHIATION OF E. COLI CELLS FOR ELECTROPORATION". USING THIS PROCEDURE, PLASMID pRIT-2T:GAG/103 WAS INTRODUCED INTO VIRULENT 245FT. PLACID CONCENTRATIONS WERE PREVIOUSLY DETERMINED TO BE STABLE, AND HAD NO ADVERSE EFFECTS ON THE VIRULENT PLASMID. THIS STRAIN WAS TESTED FOR INVASIVENESS BY A PLAQUE ASSAY DONE BY ED OAKS, AND THE STRAIN WAS ISOLATED FROM A PLAQUE. THIS ISOLATE WILL BE TESTED FOR AN IMMUNE RESPONSE IN THE MURINE-LUNG MODEL. STRAINS X602 & M41-43 ALL WERE ALSO TRANSFORMED

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6X HIS PRIMERS WERE SYNTHESIZED FOR USE IN PROTEIN
PURIFICATION OF CLONED GENES. PURIFICATION IS OBTAINED USING
Ni²⁺ BINDING RESIN (QIAGEN)

6X HIS F 5' GATC/AT CAC CAT CAC CAT CAC CAC CAC

6X HIS R 3' TTA GTG GTA GTG GTA GTG CCA CTA

MUTATED BAMBI RESTRICTION SITE. ALLOWS
CLONING TO OCCUR DOWNSTREAM OF THE 6X HIS
TAG.

VIF WAS CLONED/EXPRESSED IN THIS SYSTEM, BUT SOME
PROTEIN CONTAMINATION STILL OCCURS UPON ELUTION FROM THE
Ni²⁺ COLUMN.

Arthur A. Brantner

SUCIDE VECTORS WERE FINALLY CONSTRUCTED IN WHICH ASD
CAN BE MUTATED IN THE CHROMOSOME OF VIRTUALLY ANY GRAM
NEGATIVE BACTERIA HAVING SEQUENCE HOMOLOGY WITH E. COLI
ASD. THE SMOLEST CONSTRUCT IS THE RESULT OF A PCR
AMPLIFICATION (REVERSE) OF ASD REMOVING 552 bp FROM THE
INTERNAL SEQUENCE OF ASD, RENDERING IT NONFUNCTIONAL. WTD
THIS REGION, THE SmaI Km^r CASSETTE IS ALIBATOR. THE
ENTIRE ASD::Km^r REGION WAS PCR AMPLIFIED WITH SALI & SmaI
RESTRICTION SITES FOR CLONING INTO THE SUCIDE VECTOR pCVD442.

Arthur A. Brantner

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A *SHIGELLA* STRAIN OBTAINED FROM HANK HANK IS
 ATTENUATED FOR TNYA-1 (TNYA-2). UNFORTUNATELY, THIS STRAIN
 STILL CONTAINS THE Amp^R REGION, SO A MAMMALIAN EXPRESSION
 VECTOR CANNOT BE DIRECTLY INTRODUCED INTO THE STRAIN.

THE CONCEPT BEING THAT *SHIGELLA* COULD SERVE AS
 A CARRIER OF FOREIGN DNA, MUCH LIKE A NAKED DNA
 DELIVERY SYSTEM. THE Amp^R PLASMID pCMV3 WAS RETAINED
 FROM CLONING. 2 PLASMIDS WERE CONSTRUCTED:

→ $\text{ApB322}^{\#2A}$ WAS CONSTRUCTED BY PSTI/EORI DIGESTION TO
 REMOVE Amp^R , & RECONSTRUCTION WITH A PSTI/EORI POLYLINKER.
 THIS PLASMID WILL BE USED TO THE RECIPIENTS TE^R
 FOR SUICIDE VECTOR MATINGS.

→ $\text{ApB322}^{\#5A}$ WAS CONSTRUCTED BY PSTI DIGESTIONS
 & SUBSEQUENT LIGATION OF THE 2 VECTORS. THIS CONSTRUCT
 CAN NOW BE USED IN Amp^R BACKGROUNDS THAT ARE TE^S .

Arthur A. Branton

CONJUGATION EXPERIMENTS WITH *SHIGELLA* CONTAINING THE
 SUICIDE VECTOR $\text{pCH442}::\text{DAS}/\text{Km}^R$ WERE UNSUCCESSFUL. CONJUGATIONS
 WERE SUCCESSFULLY REFOINED WHEN THE PLASMID WAS PLACED
 INTO *SHIGELLA* & MATED WITH VIRULENT 2457T CONTAINING
 THE TE^R PLASMID $\text{ApB322}^{\#2A}$. PCR SCREENING WAS NECESSARY TO
 DETECT PRIOR INTEGRATIONS AS OPPOSED TO PLASMID MAINTENANCE
 & INTEGRATION INTO THE WRONG SITES.

Arthur A. Branton

SUBSEQUENT PCR ANALYSIS DETERMINED THAT AT LEAST 3
 CLONES WERE CORRECT, & CHECKED OUT FOR VIRULENCE PLASMID
 MARKERS. Km^R COLONIES WERE PLATED ONTO SUCROSE NAP PLATES
 AND SCREENED FOR Amp^R , OAT REQUIREMENT, & CR BINDING, & TE^R .
 2 CLONES ARE PICKED THAT HAVE ALL THE PROPER PHENOTYPES
 & GENOTYPES, 2457T, A322, $\text{DAS}::\text{Km}^R$ #15 & #19A.

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LARGE SCALE PURIFICATION OF VIF VIA GADYD GANIS RESULTED IN PROTEIN COLLECTED PREDOMINANTLY VIF, BUT OTHER BANDS AS WELL, INCLUDING WHAT APPEARS TO BE LPS THAT ELECTROPHORESIS AT THE SAME MOLECULAR WEIGHT. ELUTION CONDITIONS WERE NOT OPTIMIZED, & THE PROTEIN WAS ELUTED IN 50mM BUFFER C (DIAGON) CONTAINING 250 mM IMIDAZOLE.

Arthur A. Branstetter

GROWTH CURVES ARE PERFORMED ON STRAINS 245FI, 1322, #15C (ASO), 245FI WILD-TYPE, & MUTANT STRAINS #15C & #19A COMPLEMENTED WITH GADYD. WHILE DA'S ARE VERY COMPARABLE, THE # OF VIABLE BACTERIA PER TIME POINT VARIOUS CONSIDERABLY. IT WAS ALSO DETERMINED THAT #15C GROWN IN THE PRESENCE OF DAP HAD A 10-FOLD LOSS OF VIABILITY EVERY 30 MINUTES FOR THE FIRST COUPLE OF HOURS WHEN THE CELLS HAD BEEN GROWING LOGISTICALLY. GADYD PLS INFECTION WITH #15C DID NOT FORM CONJUGATIVES (T. HARTMAN) AND THE COMPLEMENTED STRAIN DID, THOUGH NOT TO THE EXTENT OF WILD-TYPE 245FI.

Arthur A. Branstetter

TO TEST THE POSSIBILITY OF B-GAL EXPRESSION FROM GADYD WITHIN CELL LINES VIA DELIVERY BY #15C. SINCE THE PLASMID IS INTRODUCED VIA ELECTROTRANSFORMATION IT APPEARED THAT THE STRAIN #15C WAS VERY RESISTANT TO TRANSFORMATION - DUE POSSIBLY TO THE 1322 BASED PLASMID ALREADY THERE, AND THE WEAKENED STATE OF THE DAP REQUIRING CELLS.

Arthur A. Branstetter

A Thy A-3 CONSTRUCT FROM N. HENNING WAS OBTAINED TO DETERMINE IF IT COULD SERVE AS A DELIVERY VEHICLE FOR PLASMS TO BHK CELLS. UNFORTUNATELY THIS STRAIN WAS FOUND TO BE CR- & THEREFORE WOULDN'T INVADE. HANK IS ATTEMPTING TO RECONSTRUCT THE STRAIN.

Arthur A. Branstetter

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ENL⁵⁰ WAS CLONED INTO PLASMID pJW4303 (MULLIN'S PLASMID CONTAINING TPA LEADER SEQUENCE). THIS PLASMID WAS OBTAINED FROM JOFF LYON. WE CANNOT USE THE PLASMID WITHOUT OBTAINING PERMISSION FROM AGRACORUS FIRST. THIS CONSTRUCT HAS YET TO BE TESTED FOR EXPRESSION IN MAMMARY CELLS.

Arthur A. Brantner 4/3/95

THE HYGROMYCIN GENE WAS CLONED FOR USE IN SELECTING DNA DELIVERY VECTORS IN VITRO. THE GENE WAS CLONED INTO pUC19 THEN HINDIII/SAL1 CLONED INTO pCMV. IT HAS BEEN DETERMINED THAT THE HYG CONCENTRATION FOR DHK OR PHIS CELLS WILL HAVE TO BE TITRED FOR EACH CELL LINE, & 2-FOLD DILUTIONS WILL BE TESTED (200-800 $\mu\text{g}/\text{ml}$?).

Arthur A. Brantner

A WHITE ISOLATE OF IS₁ phage WAS PICKED FROM A NOBAY STREAK OF COLS ON A TSA, IR OAP IMPROVED PLATE. THIS ISOLATE WAS FOUND TO BE IP₂D (2FI ANTIBODY) & IP₂C (2G₂ ANTIBODY) NEGATIVE. THIS STRAIN IS TO SERVE AS THE CONTROL IN THE INVASION ASSAY TO SHOW THAT UPTAKE & SUBSEQUENT EXPRESSION OF β -gal IS DUE TO THE INVASIVE PHENOTYPE OF IS₁.

Arthur A. Brantner

THE TAT-REV-EAN SEQUENCE FROM HIV₁ IS AMPLIFIED TO RNA AMPLIFY & CLONED INTO A DNA EXPRESSION VECTOR (pCMV). WHILE AMPLIFIED PRODUCT CAN BE OBTAINED, THE PRODUCT REFUSES TO BE CLONED. VARIOUS RESTRICTION SITES HAVE BEEN IMPLEMENTED, INCLUDING NOTI, NHEI, KpnI, ClaI. AFTER SUB CLONING INTO A VECTOR SUCH AS pUC18, IT APPEARS THAT THE 3' END RESTRICTION SITE IS MISSING, & THE PRIMERS APPEARS TO BE FULL LENGTH.

Arthur A. Brantner

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FOR CONSTRUCTION OF VARIOUS INSERTS INTO THE plasmid, THE VECTOR WAS DIGESTED WITH NOTI, AND PURIFIED & LIGATED BACK TO ITSELF. THIS plasmid, NOTI #1 VECTOR WILL BE USED FOR SUBSEQUENT CLONING INTO NOTI SITE. FOR AMPLIFICATION OF THE VECTOR TO GENERATE UNIQUE RESTRICTION SITES WAS NOT ALLOWED FOR THE SUBSEQUENT CLONING OF INSERT.

Arthur A. Brantley

6/20/95

THE ASD WILL BE SEQUENCED FROM BOTH S. FREEMORE & S. SOURCE FOR FURTHER INFO IN MAKING ASD MUTATIONS & TO DETERMINE IF THE S. SOURCE PROBLEMS IN GENERATING AN ASD MUTANT IS RELATED TO SEQUENCING DISCREPANCIES.

Arthur A. Brantley

7/7/95

GUINIA PIG EXENTS ARE BEING RUN FOR THE 3rd TIME TO DETERMINE IF 15g & 15g plasmid CAN PROTECT. PRELIMINARY RESULTS ARE POSITIVE, ALTHOUGH IT APPEARS THAT 15g IS MORE (SLIGHTLY - THOUGH) PROTECTIVE THAN 15g plasmid.

Arthur A. Brantley

7/25/95

THE DETERMINATION OF COPY NUMBER OF THE SALMONELLA ASD PLASMIDS WILL BE PERFORMED USING A COMBINATION OF PCR REACTIONS UTILIZING PRIMERS FOR ASD. THE USE OF REPLICATION FOR 101, & 554. THIS WILL DETERMINE COPY NUMBERS FOR plasmids 102, 103, 104, & 105. COPY # CAN ALSO BE DETERMINED FOR THOSE PLASMIDS CONTAINING INSERTS TO DETERMINE WHAT EFFECTS THOSE INSERTS HAVE ON COPY NUMBER.

Arthur A. Brantley

8/11/95

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